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ORIGINAL RESEARCH

Genetic and Neurophysiological Correlates of the Age of Onset of Alcohol Use Disorders in Adolescents and Young Adults

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Abstract Discrete time survival analysis was used to assess the age-specific association of event-related oscillations (EROs) and *CHRM2* gene variants on the onset of regular alcohol use and alcohol dependence. The subjects were 2,938 adolescents and young adults ages 12–25. Results showed that the *CHRM2* gene variants and ERO risk factors had hazards which varied considerably with age. The bulk of the significant age-specific associations occurred in those whose age of onset was under 16. These associations were concentrated in those subjects who at some time took an illicit drug. These results are consistent with studies which associate greater rates of alcohol dependence among those who begin drinking at an early age. The age specificity of the genetic and neurophysiological factors is consistent with recent studies of adolescent brain development, which locate an interval of heightened vulnerability to substance use disorders in the early to mid teens.

Keywords Alcoholism · CHRM2 · Survival analysis · ERO · Genetics · Adolescents

Introduction

That genetic factors have an age-specific influence on the onset of alcohol dependence is suggested by the findings that there are strong genetic effects contributing to risk for

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The members of Collaborative Study on the Genetics of Alcoholism (COGA) are given in Appendix.

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alcohol dependence particularly connected with early onset of drinking activity (Rangaswamy and Porjesz 2008; Sartor et al. 2009; Agrawal et al. 2009; Xuei et al. 2010; Kendler et al. 2011a, b; Lee et al. 2012). Correspondingly, the rate of adult alcohol dependence is significantly greater among those who start drinking at a relatively early age (14 years or younger) than among those who start drinking after the age of 19 (Grant and Dawson 1997) (see also Hingson et al. 2006a, b; Hussong et al. 2008; Chen et al. 2011).

Studies of adolescent brain development point to neurophysiological factors that could enhance the likelihood of substance use/abuse in those between 14 years of age and 17 years of age (Steinberg et al. 2008; Steinberg 2010a, b; Nixon and McClain 2010; Spear 2011). Significant changes in the dopaminergic system occur during adolescence, as well as growth and refinement of prefrontal and limbic circuitry (Bava and Tapert 2010; Doremus-Fitzwater et al. 2010; Galvan 2010; Koob and Volkow 2010; Naneix et al. 2012). As a result of the early enhanced activity of the mesolimbic system in contrast to the more slowly maturing prefrontal control systems and their connections to other brain regions, changes in the adolescent brain may lead to enhanced risk taking compared to earlier and later stages of maturation. Specifically, these changes may lead to a reduced cognitive control of the reward system in the brain in early to middle adolescence, leading to increased risk for alcohol and other substance abuse disorders (Casey et al. 2008; Casey and Jones 2010; Somerville and Casey 2010).

Alcohol dependence and risk for alcoholism in both adults and adolescents is associated with reduced power in event related oscillations (EROs) in a number of different experiments which elicit a P3 or P300 response (a response peak between 300 and 500 ms after the presentation of an infrequent target stimulus) (Jones et al. 2006a; Kamarajan et al. 2006; Rangaswamy et al. 2007; Patrick et al. 2006; Gilmore et al. 2010). ERO power in a task that elicits a P3 response is also associated with a number of SNPs in the *CHRM2* gene (Jones et al. 2004, 2006b).

Alcohol dependence in adults was found to be associated with a number of SNPs in the cholinergic M2 receptor gene (*CHRM2*) in two studies (Wang et al. 2004; Luo et al. 2005). A refinement of the study of Wang et al. (2004) showed that the association was present only in those subjects who had comorbid illicit drug dependence (Dick et al. 2007). This group of subjects and their family members form a genetically vulnerable group, that is, a group whose alcohol dependent members have a more heritable form of the disorder. The alcohol dependent members of this group had a significantly earlier age of onset of drinking compared to the alcohol dependent subjects without comorbid drug dependence. A generalized measure of externalizing psychopathology including alcohol dependence and illicit drug dependence is associated with the same group of SNPs in the *CHRM2* gene (Dick et al. 2008). Additionally, there is variation in the genetic factors associated with alcohol dependence; multiple genetic factors were found to contribute to a DSM-IV diagnosis of alcohol dependence in adults (Kendler et al. 2012). Some differences were found between genetic factors involved in alcohol consumption in adolescents and in young adults (Edwards and Kendler 2013) in twin study models.

In order to investigate the age specificity of the genetic and endophenotypic factors noted above on the early onset of alcohol use and dependence, we studied adolescents and young adults drawn from the Collaborative Studies on the Genetics of Alcoholism (COGA) sample (Edenberg et al. 2005). Because we wanted to understand the processes which lead from non-drinking to regular drinking to alcohol dependence we used both the onset of regular alcohol use and of alcohol dependence as dependent variables. As we noted above, more severe cases of alcohol dependence in adults were found associated with earlier ages of onset of drinking and are more likely to be the result of genetic factors, thus we hypothesized that specific genetic and related neurophysiological endophenotypes would have a greater predictive power in those with the earliest ages of onset. In particular, we decided to investigate:

1. Whether the reduced ERO measures associated with adult alcohol dependence would be significant predictors of the onset of alcohol dependence in adolescents. Specifically, whether the predictive value of these measures would be greater for the younger ages of onset than for the older ages.
2. Whether some of the same *CHRM2* SNPs associated with adult alcohol dependence would be significant predictors of the onset of alcohol dependence in adolescents. Specifically, whether the influence of these SNPs would be greater for the younger ages of onset than for the older ages of onset.
3. Whether the duration between the age of onset of regular alcohol use and the age of onset of alcohol dependence differed between different ages of onset of either regular alcohol use or alcohol dependence.
4. Whether there was there in this sample a behaviorally identifiable subsample who form a genetically vulnerable group. This would be a subsample in which the genetic effect on the onset of alcohol dependence is greater than that found in the entire sample. Such a subsample would be defined by criteria analogous to the those used in defining the genetically more vulnerable group in the COGA adult sample.

Discrete time survival analysis (DTSA) (Singer and Willett 1993; Willett and Singer 1993; Rodriguez 2007)

was used to investigate the contribution of genetic variants in *CHRM2*, ERO power, and environmental factors to the onset of regular alcohol use and of alcohol dependence in adolescents and young adults, to deal with the first two items of investigation. DTSA provides age-specific measures for the effects associated with predictive variables. Additional statistical tests, including both genetic and endophenotypic independent variables, were used to link the onset of regular alcohol use to the onset of alcohol dependence, to deal with the third item of investigation. To deal with the fourth item, the same DTSA methodology as was used for the entire sample was applied to a behaviorally defined subsample, the definition of which is discussed subsequently (see "DTSA methods" section). The results of the DTSA calculations suggested further investigation of age related changes in the genotypic distributions of those who became alcohol dependent. A further test was made to determine whether there was an effect of alcohol use on our endophentypic covariates.

Methods and materials

Subjects

Data were analyzed in a cross sectional sample ($N = 2{,}938$) of subjects who were assessed at least once when they were between the ages of 12 and 25 years. They were drawn from multiplex (densely affected) alcoholic families (recruited through a proband in treatment) and a set of community (comparison) families in the Collaborative Studies on Genetics of Alcoholism (COGA). Written informed consent was obtained from all subjects, and the Institutional Review Boards (IRB) of each collaborative site approved all procedures. The procedures used by COGA for diagnostic interviews and recording and analyzing EEG data have been described previously (Begleiter et al. 1995, 1998; Reich 1996; Edenberg et al. 2005). A detailed description of population characteristics of alcohol use and dependence are given in "Population description" section.

Clinical variables

Diagnostic measures for outcomes were taken from direct interviews using the Semi Structured Assessment of Alcoholism (SSAGA) instrument (Bucholz et al. 1994; Hesselbrock et al. 1999). Data were obtained from child (CSSAGA-I, CSSAGA-II) and adult (SSAGA-IV) versions of the SSAGA. DSM-IV criteria were used for alcohol dependence and DSM-III-R criteria were used for other substance-use related diagnoses. Once the criteria for a diagnosis were met, the diagnosis was recorded as present, regardless of any subsequent change in status as determined by succeeding interviews. The age of onset was determined from data obtained at the first interview that recorded the diagnosis as present.

Illicit drug use was defined as the use of any of heroin, cocaine, barbiturates, or amphetamines without regard to frequency or age of onset.

Genotyping

Genotyping was performed using Illumina's GoldenGate assays (Illumina, San Diego, CA, USA) at the Genome Technology Access Center at Washington University School of Medicine in St. Louis. Five *CHRM2* SNPs, rs978437, rs7800170, rs1824024, rs2061174, and rs2350786, were used in the analysis. The first three are upstream of Exon 4, the other two downstream. All of the SNPs were in Hardy–Weinberg equilibrium. The pairwise r^2 of the SNPs ranged from 0.778 to 0.141.

Electrophysiology

A substantial literature indicates that alcohol dependence and risk for alcoholism are associated with reduced levels of brain activity when subjects respond to infrequent target stimuli within a sequence of non-target stimuli (Iacono et al. 2002, 2003; Porjesz et al. 2005; Hicks et al. 2007). Representation of this response in terms of brain rhythms or EROs has proved fruitful (Rangaswamy and Porjesz 2008; Jones et al. 2006a; Gilmore et al. 2010). The ERO amplitudes used in this study were obtained from responses to rare target stimuli that elicited a P3 component in a visual oddball experiment at three midline leads (Fz, Cz, Pz). Three leads were chosen because of topographical variation in the significance of results in previous studies (Jones et al. 2006a; Rangaswamy et al. 2007). The amplitudes were calculated using the S-transform applied to the recorded data for the delta frequency band (1–3 Hz) extending from 300 to 700 ms post-stimulus. Jones et al. (2006a, b) provides a complete description of the experiment and the calculation of the values. The values were log transformed and non-parametric age regression (loess) was performed on the variables and the standardized residuals used for further analysis.

Methodology for age-specific analysis

DTSA methods

Since the principal objective is to determine whether there are age-varying effects of the predictive variables, survival analysis using standard Cox proportional hazards models in which effects are age invariant is not appropriate. In

addition, such models cannot account for differential effects on survival which are the result of unmeasured heterogeneity in the sample (frailty effects) (Wienke 2007). DTSA (Singer and Willett 1993; Willett and Singer 1993; Rodriguez 2007) provides an alternative model which avoids these problems and which can be implemented with logistic regression methods. By dividing subjects into groups based upon age of onset, a single logistic regression model can be applied to estimate the probability of those at risk in each age group of becoming alcohol dependent (or whatever other outcome is of interest) as a function of the predictive variables (covariates). The functional form of the model can be set to determine age-specific effects and/or age-independent effects, and use age-invariant and/or age-dependent covariates. A weighted model was employed to enable the use of all members of multi-member families (See "Survival analysis models" section for a more complete discussion of the DTSA model, and "Treatment of familial data and population structure" section for a detailed description of the method for calculating the weights). The output of a DTSA calculation is the same as the output from a logistic regression calculation.

Each DTSA model had the following structure: the outcomes, or dependent variables were either alcohol dependence or regular alcohol use. Regular alcohol use was defined as consumption at least once a month for 6 or more consecutive months. In all cases four distinct age ranges were used: under 16, 16 and 17, 18 and 19, over 19. These age groups were determined by the fact that ages of onset were whole numbers of years, that the numbers of those who became alcohol dependent be about the same in each group, and that there be at least 50 subjects in each group who became alcohol dependent to provide a reasonable degree of statistical reliability in the calculations. The same age grouping was used for regular alcohol use for comparative purposes. The covariates (predictive variables) were a genotype from a *CHRM2* SNP, ERO power (delta 1–3 Hz) from one of the leads, family type (multiplex alcohol family or community family), number of parents who smoke, gender, and scores on principal components 1 and 2 derived from the stratification analysis of the sample genome (see "Treatment of familial data and population structure" section). The *CHRM2* SNPs analyzed here, rs978437, rs7800170, rs1824024, rs2061174, and rs2350786 include the three most significant of those for alcohol dependence with comorbid drug dependence in Dick et al. (2007, Table 1) as well as two others that appear to be in a range of significance indicated by that table. From preliminary statistical screening of the genotypic distributions in the sample, a recessive model was employed which contrasted major allele homozygotes with those who were not. The electrophysiological phenotypes (EROs) used in the analysis were found to be significant in previous studies (Jones et al. 2006a; Kamarajan et al. 2006; Rangaswamy et al. 2007); these studies showed reduced amplitudes in alcoholics and in those offspring at high risk. The number of parents who smoke were selected in part because the Kaplan–Meier curves with different values showed considerable variation (see Keyes et al. (2008) for a discussion of the effects of parental smoking on adolescent behavior.)

DTSA results were calculated for the entire sample. Our fourth item for investigation, whether the influence of these SNPs would be greater in a behaviorally defined subsample comprising a putatively more genetically vulnerable group was suggested by the results of Dick et al. (2007) and King and Chassin (2007). Given the prevalence of various substance abuse categories in the sample and the number of subjects in each category who become alcohol dependent during the age range of the study, the broad criterion of the use of an illicit drug (see "Clinical variables" section) regardless of age of onset or frequency of use was employed to define the more genetically vulnerable group. This subsample will be called the "illicit drug use" subsample. Unlike the definition of illicit drug use in Dick et al. (2007), this definition does not categorize regular use of cannabis as illicit drug use. Since more than half the sample are characterized as regular users of cannabis at some time during the age range of the study (46 % among those from community sample), regular use of cannabis can not be considered a practice that violates norms of age-related behavior or involves enhanced risk taking, and thus not an element of "externalizing psychopathology". We note that 90 % of cannabis *dependent* subjects who are also alcohol dependent are included in the subsample, so although our criterion does not span regular cannabis use we are probably picking up those more genetically vulnerable cannabis dependent subjects and thus paralleling the group used in Dick et al. (2007). For the regular alcohol use outcome, there were a sufficient number of illicit drug non-users who became regular users of alcohol to provide a subsample to contrast with the illicit drug use subsample. Since about 75 % of the alcohol dependent subjects were members of the illicit drug use subsample, there were too few alcohol dependent subjects with no illicit drug use to provide a contrasting subsample. However some inferences about the significance of illicit drug use for the onset of alcohol dependence can be drawn from the differences between the DTSA results for the entire sample and the results for the illicit drug use subsample.

Since regular alcohol use is a necessary condition of alcohol dependence, it could not be used as a covariate in the DTSA calculation of the onset of alcohol dependence. In order to investigate the duration of the transition from regular alcohol use to alcohol dependence as a function of the age of onset of alcohol dependence, the third item for

investigation, logistic regression analyses of the onset of alcohol dependence as the outcome in each of the age ranges, restricted to the sample of those who are regular users of alcohol within that age range, were carried out. All covariates used in the DTSA calculations were used with duration of drinking as an additional covariate. (Duration of drinking was modeled both as a linear effect only and a linear and quadratic effect.) Although those who become alcohol dependent are removed from the sample at each age range, this is not a survival analysis method because new regular users of alcohol are added to the sample at each age range. However, the results of these tests can be compared to the DTSA results for the illicit drug use subsample to examine the effect of including all alcohol dependent subjects in the sample, as opposed to a restricted subsample as found in the illicit drug use subsample.

In order to investigate the duration of the transition from regular alcohol use to alcohol dependence as a function of the age of onset of regular alcohol use, both Fisher's exact test and the Cochran-Armitage trend test were applied to the distribution in each of the first three age ranges of the proportion of those who became alcohol dependent in the same or subsequent age range for those who became regular users of alcohol in that age range.

Age-related trends in genotypic distributions

We investigated whether there were age-related trends in the genotypic distributions which underlie the results of the DTSA for the SNP covariates and the rapidity of the transition from regular alcohol use to alcohol dependence. Two separate Cochran-Armitage trend tests were carried out on genotypic distributions of the SNPs of the illicit drug use subsample. Given the use of the recessive genetic model in the DTSA tests, subjects in the illicit drug use subsample were divided into two genotypic groups, those who had two copies of the major allele and those who did not. The first trend test was of the genotypic distribution of those who became alcohol dependent as a function of age of onset of alcohol dependence, comparing those who had two copies of the major allele with those who did not. The null hypothesis is that the relative effect of having a particular genotype does not vary linearly between ages of onset; that is, that the ratio of different genotypes of those who become alcohol dependent does not display a linear trend between ages of onset. (We note that the genotypic distribution of the at-risk group did not vary across age ranges.) To test whether there was trend in the genotypic distributions as a function of the rapidity of the transition from regular alcohol use to alcohol dependence, a second trend test was carried out. This test was of the genotypic distribution of those who began regular alcohol use in the youngest age range and became alcohol dependent at any age as a function of age of onset of alcohol dependence, comparing those who had two copies of the major allele with those who did not. The null hypothesis is that the ratio of different genotypes of those who become alcohol dependent does not show a trend between different time spans from the onset of regular alcohol use to the onset of alcohol dependence. We restricted our analysis to those who became regular alcohol users in the youngest age range in order to obtain results for those who might take a relatively long time to develop alcohol dependence.

Additional statistical procedures

A question of interest is whether regular consumption of alcohol affected ERO values in our sample. To examine this the residuals from the non-parametric age regression of the log transformed ERO data were used in an ANCOVA. Subjects were divided into three groups: non-drinkers ($N = 1{,}148$), drinkers from community families ($N = 304$), and drinkers from COGA families ($N = 921$). The continuous covariate was the difference between the age at test and the age at onset of drinking. In order to include the non-drinkers in this test, the difference values for them were taken from normally distributed random numbers with the same mean and variance as the difference values for the drinkers.

To further characterize the illicit drug subsample, we determined whether ERO values differed between the illicit drug subsample and its complement in the entire sample. A two sample t test was used for this purpose.

Population description

The prevalence of alcohol use and dependence in the sample being studied is shown in Table 1 in a form relevant to DTSA. In DTSA, for each outcome, those who have the possibility of suffering the outcome in each age range are the at-risk group. The at-risk group in the youngest age range is the entire sample. In each succeeding age range those who have suffered the outcome previously or for whom no information for that age range is available are removed from the at-risk group. Consequently the at-risk group diminishes in size in each successive age range. Because more subjects become regular users of alcohol than become alcohol dependent in each age range, the at-risk group for alcohol dependence is increasingly larger than the at-risk group for regular alcohol use in each subsequent age range. The illicit drug use subsample is also characterized in the table.

Table 1 Prevalence of alcohol use and dependence

	Age range (years)			
	Under 16	16–17	18–19	Over 19
Regular alcohol use				
N (at-risk = total)	2,938	1,909	1,143	496
Affected in age range	440	467	410	212
Affected with illicit drug use	266	209	116	34
Affected with rapid dependence	47	56	27	6
Proportions				
Affected in age range	0.15	0.24	0.36	0.43
Affected with illicit drug use	0.60	0.45	0.28	0.16
Affected with rapid dependence	0.11	0.12	0.07	0.03
Alcohol dependence				
N (at-risk = total)	2,938	2,264	1,784	1,229
Affected in age range	59	84	98	67
Affected with illicit drug use	45	64	64	49
Proportions				
Affected in age range	0.02	0.04	0.05	0.05
Affected with illicit drug use	0.76	0.76	0.65	0.73

N is the total number of subjects of that age range who have not previously become affected and for whom information about their status in that age range is known. This is the number at risk for that age range. Affected in age range is the number of subjects in that age range whose age of onset is within that age range. Affected with Illicit Drug Use is the number of affected subjects in age range who have ever used an illicit drug (see "Clinical variables" section for definition), regardless of frequency or age of onset. With regard to proportions, Affected in age range is the proportion of those at risk who become affected. Affected with Illicit Drug Use is the proportion of the *affected* who have ever used an illicit drug, regardless of frequency or age of onset. Affected with Rapid Dependence refers to the number and proportion of the regular alcohol users who become alcohol dependent within 1 year of the onset of regular alcohol use

Results

DTSA

For each of the five SNPs an analysis was run with the ERO measure taken from each of the three leads, as described in "Electrophysiology" section for a total of fifteen models. An examination of the logistic regression results showed that for each SNP, the beta coefficients had little difference when different leads were used; similarly, for each ERO measure the beta coefficients had little difference when different SNPs used. The same was true of coefficients for the clinical variables. We conclude that the effect of each covariate is essentially independent of the effect of any of the others. Thus results from SNPs, electrophysiological variables, and other variables can be reported seriatim without any distortion. Applying the Nyholt correction (Nyholt 2004) derived from the LD matrix, we obtain 3.2 effective SNPs. The independence of the covariates also implies that the effective number of tests is no more than the number of age ranges times the sum of the effective number of SNPs and electrophysiological variables in each sample group. Considering that the overall pattern of results is of primary interest, not only the positive results, and that no consensus exists for the most appropriate way to handle the analysis of correlated phenotypes and correlated SNPs in these circumstances, we do not enter any corrections for multiple testing. Table 2 provides all significant results for the youngest and oldest age ranges. The appendix provides complete tables for all age ranges.

Table 2 DTSA: delta ERO and *CHRM2* SNP *p* values for regular alcohol use and alcohol dependence in the entire sample and illicit drug use subsample

	Entire sample		Drug use (ever) subsample	
	Age range (years)		Age range (years)	
	Under 16	Over 19	Under 16	Over 19
Delta ERO regular alcohol use				
N at risk	2,938	496	676	50
N affected	440	212	266	34
Fz	0.003**	0.253	0.028*	0.039*
Cz	0.003**	0.512	0.050	0.031*
Pz	0.030*	0.298	0.016*	0.043*
Delta ERO alcohol dependence				
N at risk	2,938	1,229	676	365
N affected	59	67	45	49
Fz	0.001***	0.017*	0.266	0.004**
Cz	0.075	0.014*	0.840	0.011*
Pz	0.754	0.054	0.476	0.018*
CHRM2 SNP alcohol dependence				
rs978437	0.010**	0.224	0.003**	0.168
rs7800170	0.015*	0.215	0.012*	0.135
rs1824024	0.034*	0.229	0.010*	0.207
rs2061174	0.143	0.289	0.036*	0.242
rs2350786	0.156	0.492	0.021*	0.725

* $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$

Neurophysiology

In all cases, risk increased with lower ERO values. For the onset of regular alcohol use in the entire sample, ERO values were only significant for the group with earliest ages of onset, under 16 years of age. For the onset of regular alcohol use in the illicit drug use subsample, ERO values were significant for the earliest ages of onset, and weakly significant in the oldest age range, over 19 years of age.

However, the size of the at-risk group in the oldest age range is so small as to make the results of questionable relevance to the study at large.

For the onset of alcohol dependence in the entire sample, ERO values were again significant in the earliest onset age range, but not to the degree as they are for regular alcohol use. They were also significant in the oldest onset age range. For the onset of alcohol dependence in the illicit drug use subsample, ERO values were significant only in the oldest onset age range. No ERO values were significant in the drug non-use subsample.

No effects of regular alcohol use on ERO values were found, using the procedure described in "Additional statistical procedures" section.

ERO values were significantly different between the illicit drug subsample and its complement at Fz and Cz, with p values of less than 7×10^{-4}.

Genetic variants

Significant *CHRM2* SNP association were noted for the onset of alcohol dependence and were found only in the those with age of onset younger than 16. These results were obtained both in the entire sample and the illicit drug subsample. In all cases with significant results, occurrence of the major allele was the risk factor. No *CHRM2* SNPs were found to be significant predictors of the onset of regular alcohol use for any age range.

In comparing the entire sample with the subsample, the *CHRM2* effects are greater in the illicit drug use subsample than in the sample as a whole. In particular, restricting the sample to those most genetically vulnerable enables two more SNPs to become significant at the 0.05 level. If the risk of the onset of alcohol dependence as a function of genotype were as great in the drug non-users as in the illicit drug use subsample, and taking into account the lower rate of regular alcohol use in the drug non-users, there would be almost twice as many alcohol dependent subjects among the drug non-users as in fact there are.

Covariates and effect sizes

The significance of family type (whether from a multiplex (densely affected) alcoholic family or community family) and number of parents who smoke was greatest in the younger age ranges. Effects are measured in changes in logit(hazard) from baseline. When significant, SNP effects were about 1.0 for having two copies of the risk allele in the recessive genetic models, and the delta ERO effect was about 0.5 per standard deviation. When significant, the parental smoking effect was about 0.2 per smoker, the family type effect ranged from 1.0 to almost 2.0, and the gender effect ranged from about 0.5 to 1.0.

Transition between regular alcohol use and alcohol dependence

In the logistic regression analyses used to investigate the duration of the transition from regular alcohol use to alcohol dependence as a function of the age of onset of alcohol dependence, genotype was not significant in any age range in both linear and quadratic models for duration. In the linear model for duration, modeled as log(1 + duration), delta ERO values at Fz are significant in the youngest age range, and both Fz and Cz ERO values are significant in the oldest age range. ERO results are consistent with those obtained in the DTSA models. Duration was significant in the three youngest age ranges. In the quadratic model for duration, modeled as the sum of log(1 + duration) and log(1 + duration)^2, the Fz and Cz ERO values are significant only in the oldest age range. The effect of duration of drinking was significant in the three youngest age ranges with an overall U-shape in the two youngest age ranges. Since the beta value for the log(1 + duration) term is negative and the beta value for the log(1 + duration)^2 term is positive, the rising part of the U-shape masks the Fz ERO effect in the youngest age range (see Table 3).

For the tests of the rapidity of the transition from regular alcohol use to alcohol dependence as a function of the age onset of regular alcohol use, those who become regular alcohol users in the youngest age range were much more likely to become alcohol dependent either in the same age

Table 3 Onset of alcohol dependence among regular alcohol users in each age range: beta values for delta ERO and duration of regular alcohol use in linear and quadratic models for duration

Alcohol dependence				
	Age range (years)			
	Under 16	16–17	18–19	Over 19
Duration: linear				
Log(1 + duration)	−2.66	−1.77	−1.24	0.00
Fz	−0.38	0.00	0.00	−0.33
Cz	0.00	0.00	0.00	−0.33
Duration: linear and quadratic				
Log(1 + duration)	−2.32	−2.32	−0.96	0.00
Log(1 + duration)^2	1.32	0.99	0.00	0.00
Fz	0.00	0.00	0.00	−0.33
Cz	0.00	0.00	0.00	−0.34

Only covariates with at least one significant beta value are included. Non-significant beta values ($p > 0.05$) have been set to zero. Values are means across all 5 *CHRM2* SNPs used

range or the subsequent age range than those who become regular alcohol users in the age ranges 16–17 or 18–19 years (using Fisher's exact test). Viewing this from a slightly different perspective, the fraction of those who transition from alcohol use to alcohol dependence in less than 2 years in the oldest age range is much smaller than that in the youngest age range. A Cochran–Armitage trend test of this phenomenon shows a p value of less than 8×10^{-5} for the hypothesis of no trend.

Genotypic distributional tests

There were age-related trends in the genotypic distributions of those who became alcohol dependent in any of the four age ranges in the illicit drug subsample. For the first trend test, of the change of genotypic distribution with age of those who became alcohol dependent at any age, the hypothesis of no trend could be rejected at a 0.003 level for rs978437, rs7800170, and rs1824024, SNPs which were significant for alcohol dependence in the entire population, and at a 0.035 level for rs2061174 and rs2350786. This means that in those who became alcohol dependent, having two copies of the major allele was the prevalent condition for who became alcohol dependent in the earliest age range, while *not* having two copies of the major allele was the prevalent condition of those who become alcohol dependent in the oldest age range.

For the second trend test, of the change of genotypic distribution with time from initiation of alcohol use to time of alcohol dependence of those who began regular alcohol use in the youngest age range and who became alcohol dependent at any age, the hypothesis of no trend could be rejected at a 0.025 level for all of the SNPs. This means that in those who became immediately alcohol dependent, having two copies of the major allele was the prevalent condition, while in those who took the longest to become alcohol dependent, *not* having two copies of the major allele was the prevalent condition. The results are presented in Table 4. This suggests a genetic influence on the rapidity of the transition from regular alcohol use to alcohol dependence among those who become regular alcohol users in the earliest age range.

Table 4 Tests for age specificity of genotypic distributions (p values)

SNP	Age of onset trend	Use-dependence timespan trend
rs978437	0.0002	0.0004
rs7800170	0.0028	0.0205
rs1824024	0.0019	0.0018
rs2061174	0.0173	0.0012
rs2350786	0.0327	0.0017

The first column is for trend in genotypic distribution as a function of age of onset; the second column is for trend in genotypic distribution as a function of time from onset of regular alcohol use to onset of alcohol dependence for those who begin regular alcohol use in the youngest age range

Discussion

Age specificity of genetic results

The pattern of significance of the ERO and SNP factors for the onset of regular alcohol use and of alcohol dependence is different between the youngest and oldest age ranges within the entire sample, as is evident in Table 2. These differences are primarily the result of differences between the populations of regular alcohol users in the two age ranges. The proportion of the at-risk sample (alcohol non-users prior to the beginning of the age range) who become regular users of alcohol increased from 15 to 43 % between the two age ranges. Biological factors (genotype and endophenotype) are significant in both the onset of regular alcohol use and of alcohol dependence in the youngest age range. The prevalence of regular drinking in the oldest age range has eliminated the effect of the biological factors in its onset; only the onset of alcohol dependence is affected by biological factors. In the older age range, since it is likely that much of the onset of alcohol dependence is driven by past drinking, particularly since relatively few of those who become alcohol dependent in the oldest age range have been drinking for a short time, those factors which are significant for regular alcohol use in the youngest age range are significant for alcohol dependence. Furthermore, it is likely that a biologically specific subpopulation of the youngest group particularly sensitive to the effects of alcohol has been effectively eliminated from the at-risk group in the oldest age range (see the last paragraph of this section).

In the illicit drug use subsample in the youngest age range, *CHRM2* is a greater factor for the onset of alcohol dependence than in the entire sample. However, EROs are not a factor in the onset of alcohol dependence in this group. The range of ERO values in the illicit drug use subsample does not differentiate those who become alcohol dependent from those who do not, although ERO values differentiate the illicit drug subsample from their complement in the entire sample. The illicit drug use sample shows greater and more extensive genetic effects than the entire sample, since the result of selecting the illicit drug use subsample is to remove those subjects whose alcohol dependence is unlikely to be genetically affected from the analysis.

In examining the results of the logistic regression analysis of the transition from regular alcohol use to

alcohol dependence in the youngest age range, the U-shaped effect of the duration of drinking suggests the presence of two distinct factors, one a susceptibility to rapidly become dependent subsequent to the onset of regular alcohol use and the other a gradual effect of continued alcohol consumption. The masking of the ERO effect by the rising component of the duration factor suggests that ERO is associated with a long term behavior pattern involving substance abuse. The absence of a genotypic effect is the result of including all those who become alcohol dependent in the analysis, not just those in the genetically more vulnerable, as can be observed by comparing the under 16 results between the regular alcohol user group and the illicit drug user group.

In summary, for the youngest age range the pattern of significance of the ERO and SNP phenotypes for the onset of regular alcohol use and of alcohol dependence, as well as the pattern of significance in the transition from alcohol use to alcohol dependence suggests that delta ERO value indexes an element of propensity to use drugs to excess, while the *CHRM2* SNPs index an age related effect of alcohol consumption on the brain with the behavioral outcome of dependence, as we explain below.

We view the age-varying genotypic effect of the *CHRM2* SNPs as an instance of a gene–environment interaction. In our case the immediate genotypic effects are upon the activation level of the type 2 muscarinic receptors and the environment is the neuroanatomic and neurophysiological context in which the action of the muscarinic receptors is taking place. This environment undergoes significant changes as the brain develops from the early teens into the early twenties, as we have noted above. In the transition from alcohol non-use to regular use of alcohol to alcohol dependence, we note that alcohol consumption has significant effects on the development of addiction in adolescent animals (Guerri and Pascual 2010; Philpot and Kirstein 2004; Maldonado-Devincci et al. 2010; Pascual et al. 2009; Coleman et al. 2011) and humans (Alfonso-Loeches and Guerri 2011; Koob and Volkow 2010; Guerri and Pascual 2010; Bava and Tapert 2010; Bava et al. 2009; Squeglia et al. 2009). The cholinergic M2 receptor gene belongs to a family of muscarinic acetylcholine G-protein coupled receptors with five known subtypes (M1–M5). The M2 receptors in the mesolimbic dopaminergic system play a significant role in modulating the level of dopamine release (Picciotto et al. 2012; Scarr 2012; Cachope et al. 2012; Oldenburg and Ding 2011; Witten et al. 2010). This has a important effect in governing the reward system (Mark et al. 2011; Shabani et al. 2010), including modulating the effects of alcohol on it (Adermark et al. 2011). M2 receptors also modulate synaptic transmission in cortical circuits affecting the pyramidal neurons (Picciotto et al. 2012).

It is not possible to determine the precise nature of the interaction between the genotypic effect on the cholinergic M2 receptors and the age-varying neuroanatomic/neurophysiologcial environment given the data at our disposal. Given the age-related patterns of genotypic action we have described above, it is possible that the effect of alcohol consumption on the brain varies with the genotype of the cholinergic M2 receptors and the age of onset of regular drinking. Specifically, when alcohol is consumed regularly in the youngest age range, perhaps better described as a particular stage in brain maturation centered in this age range, the addiction producing effects on those who have two copies of the major allele are accelerated compared to those who do not, leading to rapid transition from regular alcohol use to alcohol dependence. [This may be in part responsible for the "telescoping of trajectory" effects reported in Hussong et al. (2008).] Those without two copies of the major allele may take longer to manifest the effects of alcohol use. As the age of the initiation of alcohol use increases, it appears that the cumulative risk for alcohol dependence when carried into the adult years is greater in those without two copies of the major allele than in those with two copies. We draw this last conclusion on the basis of the trend tests on our own data and the results of the studies of Wang et al. (2004) and Dick et al. (2007). In those who become regular users of alcohol under the age of 16, a majority of those who became alcohol dependent within two years had the risk genotype; the majority of those who become alcohol dependent four years or more after their onset of regular drinking did not have the initial risk genotype.

A contributing factor to the age specificity of the effect of the *CHRM2* SNPs could be a frailty effect. The frailty effect would play a role if there were relatively easy access to alcohol in the youngest age range, at least for those most at risk. Among those who have the major alleles, those who are genetically most vulnerable become alcohol dependent rapidly, leaving only those who have some (unmeasured) protective factor(s). Thus risk for those with the major alleles will decrease with age, since those without the protective factors will have become alcohol dependent, leaving primarily those with protective factors at potential risk. We also note that if the illicit drug user population had easier access to alcohol than the entire population as a whole, the greater genetic effects seen in the illicit drug user subsample might in part be the result of a gene–environment interaction, akin to those described in Dick and Kendler (2012), in which looser social controls over behavior accentuate genetic effects. Since 80 % of the illicit drug use subsample are from COGA rather than

community families, this is a plausible hypothesis. The specific environment of the most vulnerable group is more likely to accentuate genetic effects, rather than to diminish them.

Relation to previous *CHRM2* findings

We found that SNPs reported to be significant in adults were significant in adolescents in this sample, particularly for those in the youngest age ranges, and for those who had ever used an illicit drug. However, in our results, the major allele was the risk allele, while in the results of Wang et al. (2004) and consequently of Dick et al. (2007), the minor allele was the risk allele. Our results do not contradict those of Wang et al. (2004) and Dick et al. (2007); the results are mutually consistent. Instead, they reveal a novel age-specific risk factor undetectable by solely examining the condition of alcohol dependence rather than its age of onset.

In view of the age differences between the sample studied in this paper, and the sample used in the studies of Wang et al. (2004) and Dick et al. (2007) it is not possible that they should contradict one another. In the Wang et al. (2004) study, about 5 % of the alcohol dependent subjects had ages of onset of less than 16 years of age. This is too small a fraction to have an effect on the results. As we noted in our discussion of the trend tests, in our study the genotypic distributions of the alcohol dependent subjects change with age of onset. While we do not observe a significant SNP effect in the oldest age range with DTSA, the fraction of subjects with the minor allele in those who become alcohol dependent is greater than the fraction of subjects with the minor allele in those who do not become alcohol dependent (Fisher's exact test gives $p = 0.07$ for the null hypothesis). This trend acts to produce a similar genotypic distributions for alcohol dependent and non alcohol dependent subjects when considered regardless of age of onset.

In terms of the methodology, DTSA requires that there be differences in genotypic distributions between alcohol dependent and non alcohol dependent subjects to give a statistically significant results for a SNP; this is not true for the family based method (pedigree disequilibrium test) used by Wang et al. (2004). (In that study there is no difference in genotypic distribution between the alcoholic and non-alcoholic subjects.) Our interpretation is that family based studies are more powerful than the type of association study employed here; the absence of a distributional difference does not mean that there is no genetic effect.

Relation to previous ERO findings

In the age ranges and samples in which we found that ERO was significant for the onset of alcohol use or alcohol dependence, it was the lower values which characterize the risk factor, which is consistent with the results in adolescents and young adults in the studies by Rangaswamy et al. (2007), Kamarajan et al. (2006), and Gilmore et al. (2010). In those investigations high risk groups had lower ERO values than the low risk groups.

That no effects of regular alcohol use on ERO values were found is consistent with similar results obtained by Perlman et al. (2009).

Comparison with other genetic studies of adolescents and young adults

It is important to note that the objectives of the twin studies considered here (Rose et al. 2001a, b, 2004; Iacono et al. 2003; Pagan et al. 2006; Hicks et al. 2007; Kendler et al. 2008; van Beek et al. 2011; Baker et al. 2011) and of this study are quite different. The twin studies investigate the presence of a "disease" condition, although exactly which condition varies considerably among studies. (Of the outcomes in the nine twin studies cited above, four had drinking amounts, either as frequency amounts or levels without consideration of abuse symptoms; four had alcohol abuse symptoms, one as a count variable and the others as binary, and one had intoxication levels.) The objective of this study, as a survival analysis, is to analyze the factors contributing to an event, the onset of a condition. Once the condition has come to pass, it is not of further interest in survival analysis. The genetic effects which produce the condition are only significant at the onset of the condition, and their effects persist only if the subsequent onset of the condition in other subjects is attributable to them. In the twin studies post-onset presence of the condition is part of the outcome analyzed. That is, in the longitudinal studies using multistage models, the affected subjects are retained throughout the study subsequent to their becoming affected, while in the survival analysis method used in this study, the affected subjects are removed from consideration in the study once they have become affected, and no longer influence the results. Therefore, although the use of a longitudinal multi-stage model in van Beek et al. (2011) and Baker et al. (2011) enables genetic influences to have age-specific characteristics, these effects are modeled as persisting through time as a result of an effect at a single age range.

If early onset alcohol use is associated with the more genetically determined form of alcoholism (Pickens et al. 1991; Johnson et al. 1998; Dick et al. 2007; Chen et al. 2011) then it would be expected that genetic factors leading to early drinking and dependence would be manifest. Our results are consistent with this hypothesis. The pattern of genetic results obtained here, albeit from a single gene, is weighted towards the strongest effects manifesting

themselves in the youngest age range. However, most twin studies find low genetic influences at younger ages and increases in genetic influences with age (Bergen et al. 2007; Kendler et al. 2008; van Beek et al. 2011), although not all twin studies have this conclusion (Hicks et al. 2007; Baker et al. 2011). These results can be understood after examination of the populations from which the twin samples are drawn and the outcomes which are modeled. The samples in the twin studies are drawn from the general population, not from the densely affected families which form the bulk of the sample used here. Thus genetic effects will be more difficult to find in the twin studies, particularly for the rarer, more genetically affected conditions. In a number of studies outcome definitions are broad, and are not subject to as strong genetic effect as more restricted outcomes such as alcohol dependence or externalizing disorders. The most dramatic example of this is the difference between the cross-sectional results from the Minnesota twin studies (Iacono et al. 2003; Hicks et al. 2007) in which the outcomes are narrowly defined and the cross-sectional results from a Dutch twin study (van Beek et al. 2011) with the very broad outcome of having one or more alcohol abuse symptoms. The Minnesota twin studies have $A > 0.6$ for ages 11 and 17, while the Dutch twin study has $A < 0.3$ for ages 15–17 and 18–20, where A is the additive genetic effect.

Conclusion

This study is the first to identify age-specific effects of particular genetic and neurophysiological factors on the age of onset of alcohol dependence during adolescence and young adulthood. On the basis of this study we can conclude that:

- Although the risk allele for the onset of alcohol dependence in young adolescents differs from that in adults, the results obtained are consistent with adult studies of the role of *CHRM2* in alcohol dependence. We see a gene–environment interaction in which the process of brain maturation alters the effect of genetic variants.
- The results obtained are consistent with recent studies of adolescent brain development and their consequences for adolescent behavior. These studies emphasize a "window of vulnerability" in early adolescence for sensation-seeking to result in risk-taking behavior, including substance use and abuse. The results suggest that ERO values index some aspect of risk-taking behavior and that there is a genetically affected neurophysiological window of vulnerability to the effects of alcohol consumption leading to addiction.
- The age-specificity of the *CHRM2* and ERO factors, particularly the rapidity of transition from alcohol use to alcohol dependence among the most vulnerable, has consequences for treatment strategies, suggesting the importance of early intervention in high risk groups (Casey and Jones 2010; Tripodi et al. 2010).

Clearly future research would use a longitudinal design, obtain more environmental and behavioral/clinical data, and use more sophisticated modeling, particularly the use of multiple genetic factors (Culverhouse et al. 2011). If age-specific effects are to be found, a model which can identify them applied to a sample in which they are prevalent is necessary.

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Appendix: Methodological details

Survival analysis models

Survival analysis models may be distinguished by assumptions made about the effects of the covariates on the hazard. Some models assume that these effects are time-invariant while others enable the estimation of time-varying effects, as well as the use of time-varying covariates.

The hazard function $\lambda(t)$ is defined as the instantaneous rate of the occurrence of the event.

A commonly used survival model is the Cox proportional hazards model,

$$\log(\lambda_i(t|\mathrm{x_i})) = \alpha_0(t) + \mathrm{x_i'}\beta$$

where x_i is the vector of covariates for individual i (x_i' is the transpose of x_i), β is the vector of coefficients to be estimated and $\alpha_0(t)$ represents a time-varying baseline hazard.

The assumption in this model is that the hazard due to the covariates is constant over time, in other words, that the effect of a covariate does not change over the interval studied. It is possible to extend this model to enable time-varying effects by substituting $\beta(t)$ for β in the original model. To model time-varying effects, it may be easier for computational purposes to use a discretized model for log ($\lambda(t)$), which enables piecewise estimation of the effects of covariates. Estimates of the parameters could be made using a Poisson log-linear model (Rodriguez 2007). An alternative strategy, DTSA, is to use a discretized model for logit($\lambda(t)$).

The discrete time survival model is

$$\mathrm{logit}(\lambda_i(t_j|\mathrm{x_i})) = \alpha_j + \mathrm{x_i'}\beta_j$$

with j ranging over the time intervals. We use

$$\mathrm{logit}(\lambda_i(t_j|\mathrm{x_{ij}})) = \alpha_j + \mathrm{x_{ij}'}\beta_j$$

to account for the possibility of time-varying covariates and time-varying effects.

The DTSA model parameters can be calculated by creating pseudo-observations, as many for each individual as there are time ranges starting from the first range to the one in which the outcome or censoring occurs. Each pseudo-observation contains covariate information corresponding to the form of the model, in terms of time-invariant and time-varying parameters used. Parameters are estimated by standard logistic regression algorithms (Singer and Willett 1993, 2003a, b; Willett and Singer 1993; Rodriguez 2007).

Treatment of familial data and population structure

Since most of the subjects in the study are from multi-member families it is necessary to account for correlations in the phenotypic data which arise from common genetic and environmental factors within families, and also to account for population stratification. As in a number of other recent papers (Kang et al. 2010), we use genetic relatedness information to model the covariance structure of the phenotypic data. We base our treatment of this problem on the exposition of the generalized estimating equations (GEE) method found in Liang and Zeger (1993) and the more detailed explanation of Hanley et al. (2003) of GEE model construction, and a similar approach based on pedigree information (Yang et al. 2011). The methodology of GEE is to form a weighted regression model in which the weights are a function of the covariance structure of the phenotypic data estimated from the data itself. In the method proposed here, the weights are instead estimated from the genetic relatedness structure of the subjects.

The method is as follows: Given a large enough set of SNPs from the sample, no pair of which is in linkage disequilibrium (LD), the allelic frequency for each SNP is determined. Then the pairwise relationship between all members of each multi-member family is calculated using the algorithm of Choi et al. (2009). This is equivalent to constructing a block-diagonal version of the kinship matrix Φ (with elements ϕ_{ij}) (Choi et al. 2009, Eq. 3), with the inbreeding coefficients assumed to be zero. This matrix corresponds to the variance-covariance matrix of the phenotypic data as used in the GEE method. The weights assigned to each individual in the regression model in the following manner: Each individual who is not a member of a multi-member family is assigned weight 1. Suppose individual is member i of family with n members $1, \ldots, n$. Then the weight assigned to that person is $1/(1 + 2\sum_{j=1}^{j=n} \phi_{ij,\, i \neq j})$. This corresponds to the determination of weights in the GEE model (Hanley et al. 2003).

Population stratification was dealt with by using the principal component scores derived from the complete kinship matrix Φ as additional independent variables in the regression analysis. This was found to be a satisfactory method in Astle and Balding (2009).

Complete DTSA results for delta ERO and *CHRM2* SNPs for regular alcohol use and alcohol dependence in both the entire sample and illicit drug use subsample are found in Tables 5 and 6.

Table 5 DTSA: delta ERO p values for regular alcohol use and alcohol dependence in the entire sample and illicit drug use subsample

	Entire sample				Drug use (ever) subsample			
	Age range (years)				Age range (years)			
	Under 16	16–17	18–19	Over 19	Under 16	16–17	18–19	Over 19
Regular alcohol use								
N at risk	2,938	1,909	1,143	496	676	402	186	50
N affected	440	467	410	212	266	209	116	34
Fz	0.0034**	0.6743	0.2380	0.2531	0.0284*	0.8880	0.4478	0.0387*
Cz	0.0025**	0.6665	0.2481	0.5129	0.0504	0.7952	0.6660	0.0309*
Pz	0.0302*	0.8768	0.3055	0.2981	0.0165*	0.9762	0.8040	0.0430*
Alcohol dependence								
N at risk	2,938	2,264	1,784	1,229	676	619	520	365
N affected	59	84	98	67	45	64	64	49
Fz	0.0009***	0.2946	0.8930	0.0168*	0.2657	0.4570	0.9366	0.0041**
Cz	0.0751	0.2102	0.1599	0.0137*	0.8401	0.3361	0.3377	0.0107*
Pz	0.7542	0.2309	0.0668	0.0540	0.4765	0.3370	0.2389	0.0183*

* $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$

Table 6 DTSA: *CHRM2* SNP p values for alcohol dependence in the entire sample and illicit drug use subsample

	Entire sample				Drug user (ever)			
	Age range (years)				Age range (years)			
	Under 16	16–17	18–19	Over 19	Under 16	16–17	18–19	Over 19
N at risk	2,938	2,264	1,784	1,229	676	619	520	365
N affected	59	84	98	67	45	64	64	49
rs978437	0.0099**	0.4813	0.6364	0.2242	0.0033**	0.1870	0.5125	0.1678
rs7800170	0.0150*	0.3305	0.3129	0.2147	0.0117*	0.1900	0.2042	0.1353
rs1824024	0.0343*	0.7756	0.9757	0.2290	0.0102*	0.8494	0.4215	0.2066
rs2061174	0.1427	0.6733	0.4787	0.2891	0.0364*	0.8665	0.6456	0.2421
rs2350786	0.1564	0.4349	0.6745	0.4918	0.0206*	0.2863	0.5326	0.7249

The values are for the recessive model

* $0.01 < p < 0.05$; ** $0.001 < p < 0.01$; *** $p < 0.001$

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